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Major Histocompatibility Complex Class I Genes in Murine Fibrosarcoma IC9 Are Down Regulated at the Level of the Chromatin Structure

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The fibrosarcoma IC9 is deficient in the expression of the major histocompatibility complex class I genes K^b, K^k, and D^k and expresses only the D^b molecule. Because class I deficiency may enable tumor cells to escape the immune response by cytotoxic T lymphocytes, we investigated why the class I genes are not expressed. Expression of the silent class I genes could not be induced, but all known DNA-binding factors specific for class I genes could be detected in nuclear extracts of IC9 cells. After cloning of the silent K^b gene from the IC9 cells and subsequent transfection of this cloned K^b gene into LTK$^-$ and IC9 cells, normal K^b antigens were expressed on the cell surface of both cell lines. Digestion of the chromatin of IC9 cells with micrococcal nuclease and DNase I showed a decreased nuclease sensitivity of the silent class I genes in comparison with active genes and the absence of DNase I hypersensitive sites in the promoter region of the silent D^k gene. These findings demonstrate that class I expression is turned off by a *cis*-acting regulatory mechanism at the level of the chromatin structure.

Almost all cells of mammals express class I antigens of the major histocompatibility complex (H-2 in mice) on their surfaces, albeit in various amounts (12). These class I antigens are crucial for an immune response of cytotoxic T lymphocytes against foreign antigens. Thus, cytotoxic T lymphocytes can recognize cells infected with virus or altered by neoplastic transformation only in association with major histocompatibility complex class I antigens (40). It is therefore conceivable that the control of tumor growth by the immune system depends on the presence of class I antigens on the cell surface. In agreement with this is the observation that many tumors of different origins are deficient in the expression of one or more class I antigens (14, 31). A notable example is the methylcholanthrene-induced T10 sarcoma from ($H\text{-}2^b \times H\text{-}2^k$)$F_1$ mice (8). In normal cells of F_1 mice, the alleles of the *K* and *D* class I loci are, in general, coexpressed. However, the metastatic T10 fibrosarcoma expressed only the D^b and D^k molecules, and the IC9 subline derived from it was found to express D^b but not K^b, K^k, or D^k (8, 33). In previous investigations it was shown that expression of a transfected K^b gene drastically decreased the tumorigenicity of the IC9 cells in immunocompetent syngeneic mice (33), demonstrating the importance of class I antigens for tumor rejection. Here we report that in the IC9 cells, the silent class I genes are down regulated, not by a *trans*-acting mechanism, but at the level of the chromatin structure. This mechanism not only distinguishes between different class I gene loci but also discriminates between different alleles.

Expression of the silent class I genes is not inducible. Southern blot analysis with specific oligonucleotides revealed that in the IC9 cells, all three silent class I genes (K^b, K^k, and D^k) were present on restriction fragments, with sizes comparable to those in the genome of cells positive for the respective H-2 antigens (results not shown). Therefore, it was unlikely that deletions or larger rearrangements were the cause of the lack of expression. Next, we examined whether expression of the silent class I genes was inducible. Gamma interferon (IFN-γ) can induce or enhance the expression of numerous genes, including class I genes (27). To investigate the effect of IFN-γ on the expression of class I genes in the IC9 fibrosarcoma, the cells were incubated for 48 h with 100 U of IFN-γ per ml. However, this treatment did not induce the surface expression of any of the three silent class I antigens. Expression of the active D^b gene was enhanced by a factor of approximately 2, demonstrating that the cells were responsive to IFN-γ (Table 1). There are numerous reports in which a correlation between DNA methylation and gene expression has been described (10). The cultivation of cells in the presence of the base analog 5-azacytidine leads to inhibition of methylation, which can result in the transcription of formerly inactive genes (10). To test the influence of methylation on the expression of the silent class I genes, IC9 cells were incubated for 10 days in medium containing 5 μM 5-azacytidine. In comparison with results for the negative control, in which an irrelevant anti-K^d antibody was used, the amount of D^b expression was not increased and the expression of none of the three silent class I genes was induced (Table 1). The slightly higher values obtained after treatment with 5-azacytidine were probably caused by increased nonspecific binding of antibodies to dead cells. These were caused by the toxic effects which 5-azacytidine exerted on IC9 cells even at low concentrations. We also determined the methylation pattern of the silent class I genes with methylation-sensitive restriction enzymes. However, although we used probes for various regions of the class I genes (e.g., pH-2^d-4 [20], a K^b-specific oligonucleotide [24], and pD^kRVS [see below]), we could not detect differences in comparison with H-2-positive cell lines (EL4, L929) (results not shown).

Somatic hybrids between IC9 cells and an H-2-positive cell line. The lack of expression of the three silent class I genes

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TABLE 1. Expression of class I antigens in IC9 cells

Cell line	Treatment	cpm obtained by H-2 antibody[a]					
		D^b	D^k	K^b	K^k	K^d	D^q
IC9		3,824	541	698	585	689	
IC9	IFN-γ (100 U/ml, 2 days)	8,517	567	753	542	712	
IC9	5-azacytidine (5 μM, 10 days)	2,367	956	786	1,032	1,256	
1T22-6		720	504	458	774	592	16,326
IC9/1T-1[b]		4,102	524	780	918	696	14,140
IC9/1T-2		2,228	536	900	804	584	7,504
1T22-6K^k		802	1,267	572	32,070		10,878
IC9/1TK^k-1[b]		1,904	1,170	764	26,063		14,041
IC9/1TK^k		2,668	1,034	783	31,720		5,804

[a] Values are means of triplicates obtained in a cellular radioimmunoassay (33) with monoclonal antibodies directed against H-2 antigens, anti-D^b, hybridoma B22-249 (22); anti-K^b, K10-56, (15); anti-D^k, 15-5-5S (26); anti-K^k, H100-27.55 (22); anti-K^d, K9-18 (2), and anti-D^q, T19.191. T19.191 (IgG2a/kappa) reacts with D^q, D^d, and H-2$^{\wedge}$ antigens (N. Tada, S. Kimura, U. Hämmerling, and G. J. Hämmerling, unpublished data). Underlined values are positive in comparison with values for the negative control (K^d) (backgrounds not subtracted).

[b] Cell lines IC9/1T and IC9/1TK^k are hybrids generated by fusion of IC9 with the 1T22-6 fibroblast line (*H-2^q*) and by the 1T22-6 line transfected with a K^k gene, respectively.

could be due to the absence of a positive factor which is required for class I gene expression. In that case, fusion with an H-2-positive cell line should restore the expression of the silent class I genes (14, 19). On the other hand, the presence of a (*trans*-acting) suppressive mechanism in IC9 cells should decrease or turn off class I expression in somatic hybrids with an H-2-positive cell line (3, 11). To test these possibilities, somatic hybrids between the IC9 cells and the class I-positive fibroblast line 1T22-6 (*H-2^q*, thymidine kinase negative) which had been transfected with the neomycin resistance gene alone or together with a K^k gene (cell line designated 1T22-6K^k) (2) were prepared. Table 1 shows four characteristic examples for hybrid cell clones which had been selected in medium containing hypoxanthine, aminopterin, and thymidine plus G418. As can be seen, there was neither an induction of expression of the silent (IC9) class I genes nor significant suppression of the expression of the 1T22-6-derived class I genes and the transfected K^k gene. These findings suggest that there is no dominant negative *trans*-acting factor in the IC9 cells and that the putative positive *trans*-acting factors provided by the 1T22-6 parent are not sufficient for class I induction.

Detection of DNA-binding factors specific for class I genes. Recently several factors have been described which bind to the overlapping enhancer-interferon response sequence located between −193 and −137 upstream of the transcription start site of class I genes (16, 28, 29, 39). This region has been described as the target for both positive (18) and negative regulation (25). By using a gel retardation assay, we examined whether these factors were also present in nuclear extracts of IC9 cells. Nuclear extracts were prepared by the method of Dignam et al. (9), whereby all buffers contained 1 mg of aprotinin (Sigma Chemical Co.) per ml and 0.5 ml of phenylmethylsulfonyl fluoride. The probe used for the gel retardation assay was a 95-base-pair (bp) *Dde*I-*Ava*II fragment containing the K^b enhancer and the interferon response sequence (−215 to −120, as sequenced by Kimura et al. [18]). The probe was end labeled by using polynucleotide kinase (Boehringer Mannheim Biochemicals) and [γ-^{32}P]

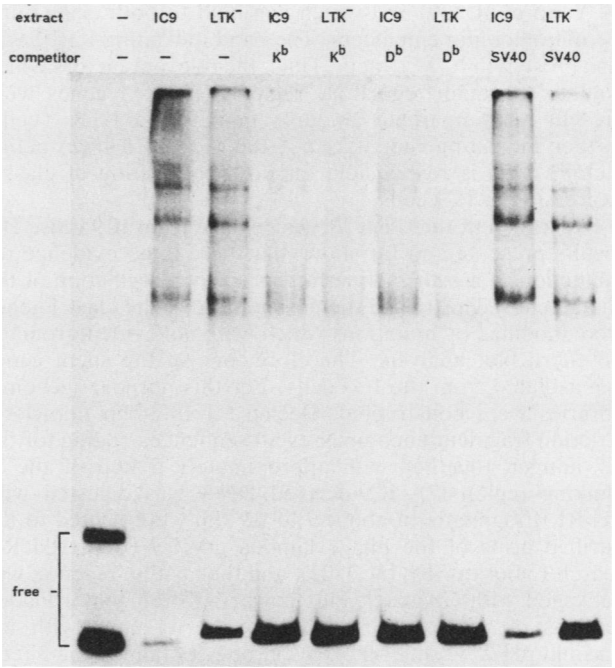

FIG. 1. Detection of DNA-binding factors specific for class I genes in IC9 and LTK$^-$ cells. Equal amounts of nuclear extracts of IC9 and LTK$^-$ cells were incubated with a γ-^{32}P-labeled *Ava*II-*Dde*I fragment of the K^b promoter carrying the overlapping enhancer-interferon response sequence, run on a 5% polyacrylamide gel and autoradiographed. As unlabeled competitor, the following DNA samples were used: K^b, the *Dde*I-*Ava*II fragment described above; D^b, a 400-bp *Xba*I-*Sma*I fragment containing the promoter region; (SV40), simian virus 40, a 270-bp *Hin*dIII-*Pvu*II fragment containing the simian virus 40 promoter, enhancer, and origin. The two bands of the free probe represent two different conformations of the same DNA fragment which are sometimes generated during preparation of the labeled probe. The upper band disappears after the free probe is heated at 65°C, without affecting the observed pattern of retarded fragments.

ATP. The binding reaction was performed with 5 μg of nuclear extracts for 30 min at room temperature. The binding buffer was as described elsewhere (16) and contained 10 μg of poly(dI-dC) (Boehringer Mannheim) as nonspecific competitor. Electrophoresis was performed through low-ionic-strength polyacrylamide gels (5%) in 0.5× TBE (1× TBE is 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). Three main binding activities appeared with nuclear extracts of both the H-2-positive LTK$^-$ and the H-2-negative IC9 cells (Fig. 1). The intensity of the shifted bands could be diminished only by addition of unlabeled competitor DNA of the regulatory region of class I genes (K^b or D^b) and not by addition of DNA of the simian virus 40 regulatory region, indicating class I specificity. The three binding activities observed here correspond to those described for H-2-positive cells (29). With extracts of LTK$^-$ cells, an additional minor band can be seen below the fastest-migrating major band. However, this band is found in only some H-2-positive cell lines (29). Thus, this binding activity is obviously not necessary for class I expression, and the class I deficiency in IC9 cells cannot be attributed to its absence. The slowest-migrating band, which was absent in extracts of the H-2-negative F9 embryonal carcinoma cells (29), was present in IC9 extracts. The fastest-migrating band indicates the presence of KBF1, a transcription factor which has been purified

by Yano et al. (39) and which can bind to both class I and β_2-microglobulin enhancers. The band indicating KBF1 was also present in IC9 extracts. Thus, the relevant factors which bind to the main regulatory region of class I genes were present in comparable amounts in IC9 and LTK$^-$ cells, further indicating that it is not the lack of a *trans*-acting factor which is responsible for down regulation of class I expression in IC9 cells.

Expression of the silent K^b gene cloned from IC9 cells. The results presented so far show that there is no evidence for the action of a *trans*-acting factor in down regulation of the silent genes. This raised the possibility that the class I genes were modified by mutations which were not evident from the Southern blot analysis. Therefore, one of the silent genes was isolated from the IC9 cells. For this purpose, genomic libraries were constructed. Because a 10.2-kbp *Eco*RI restriction fragment encompasses all sequences coding for the K^b antigen together with approximately 5 kbp of the 5′ flanking region (7), IC9-derived DNA was digested with *Eco*RI. Fragments of about 9 to 12 kbp were ligated to the purified arms of the phage lambda gtWES (Bethesda Research Laboratories, Inc.) (21), and the ligation reaction was packaged with Gigapack Plus extracts (Stratagene). Plaque replicas on nitrocellulose filters were screened with the plasmid pH-2^d-4, an H-2 cDNA probe containing the 3′ end of the coding region of the K^d gene (20). Hybridization with all plasmid probes was at 65°C in 4× SET (1× SET is 0.15 M NaCl, 20 mM Tris hydrochloride [pH 7.8], 1 mM EDTA)–10× Denhardt solution–0.5% sodium dodecyl sulfate (SDS)–300 μg of denatured calf thymus DNA per ml. Filters were washed at 65°C for 60 min in 2× SET–1% SDS and for 20 min in 0.2× SET–1% SDS. The positive phages were rescreened with a K^b-specific oligonucleotide which codes for amino acids 150 to 157 in the α-2 domain of the K^b molecule (5′-CTG AGT CTC TCT GCT TCA CCA GC-3′ [24]). Oligonucleotide hybridizations were with 10 ng of [α-^{32}P]dCTP-tailed oligonucleotide (6) at 62°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS–10× Denhardt solution–300 μg of denatured calf thymus DNA per ml–100 μg of poly(dC) per ml. Filters were washed in 6× SSC–1% SDS for 30 min at 40°C and for 5 min at 69°C. The final wash was for 5 min in 4× SSC–0.5% SDS at 65°C. The 10.2-kbp *Eco*RI insert of a positive phage was cloned into the plasmid pUC8 (32) and subjected to restriction analysis. By comparing the restriction sites of the IC9 K^b gene with the known sequences and restriction sites of the K^b gene from C57BL/10 mice (7, 18, 36), no differences could be found in the structural gene, in the 5′ regulatory region, or in the more distant 5′ flanking region (data not shown).

Because the restriction analysis did not reveal any alterations, the isolated K^b gene was transfected into LTK$^-$ cells and also into the IC9 cell line from which it was derived. The plasmid carrying the 10.2-kbp *Eco*RI fragment with the K^b gene was cotransfected with a DNA fragment carrying the neomycin resistance gene (5) by the $CaPO_4$ procedure (38). G418-resistant cells which arose were cloned and screened for cell surface expression of K^b antigens in a cellular radioimmunassay (33). K^b-positive clones could be derived from both LTK$^-$ and IC9 transfectants. Control transfections with the neomycin resistance gene alone did not yield any K^b-positive IC9 clones (not shown), and there was no induction of the K^k or the D^k gene in K^b-transfected IC9 clones (Table 2). In Table 2, class I antigen expression is shown for six different K^b-positive IC9 clones and for four examples of K^b-positive LTK$^-$ clones. It was striking that in all IC9 clones, the transfected K^b gene was expressed to a lower extent than in the LTK$^-$ clones. However, IC9 and LTK$^-$ transfectants and the strongly K^b-positive EL4 cell line contained comparable amounts of K^b-specific mRNA (Fig. 2A), suggesting that the differences in cell surface expression are due to posttranscriptional regulation (preparation of cytoplasmic RNA and transfer to nitrocellulose membranes was as described previously [37]; hybridization of RNA with oligonucleotides and plasmid probes was as

TABLE 2. Expression of the isolated K^b gene

Cell clone[a]	cpm obtained by H-2 antibody[b]				
	D^b	D^k	K^b	K^k	K^d
EL4	15,374	641	17,780	675	442
IC9	3,824	541	698	585	689
IB39	5,542	947	4,939	1,122	1,014
IG3	3,327	684	3,530	547	628
IB22	3,702	614	1,668	836	546
IB34	3,209	873	1,517	850	916
IB6	3,824	696	1,395	834	705
IB2	4,588	504	1,157	602	531
LTK		5,279	810	18,012	879
LG1		9,548	15,350	22,990	738
LG9		4,442	29,012	5,403	764
LB18		2,815	19,922	3,696	1,086
LB19		2,620	10,952	3,688	1,048
IB39+	7,438	998	8,540	1,078	860
IB22+	5,174	474	4,473	388	372

[a] IB39, IG3, IB22, IB34, IB6, and IB2 are cloned colonies of IC9 cells transfected with the K^b gene isolated from IC9 cells. LG1, LG9, LB18, and LB19 are cloned colonies of LTK$^-$ cells transfected with the K^b gene isolated from IC9 cells. IB39+ and IB22+ are IC9 transfectants treated for 48 h with IFN-γ (20 U/ml).

[b] Values are means of triplicates obtained in a cellular radioimmunoassay with monoclonal antibodies directed against H-2 antigens (see Table 1). Underlined values are positive in comparison with values for the negative control (K^d) (backgrounds not subtracted).

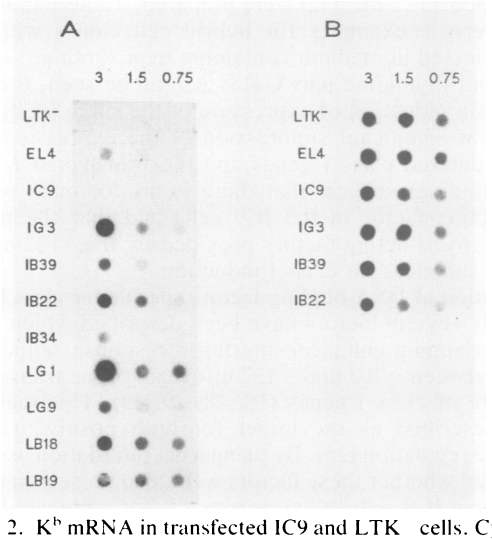

FIG. 2. K^b mRNA in transfected IC9 and LTK$^-$ cells. Cytoplasmic RNA of 3×10^5, 1.5×10^5, and 0.75×10^5 cells was transferred to nitrocellulose membranes (37). (A) Hybridization to the K^b-specific oligonucleotide; (B) Hybridization to a 2-kbp *Bam*HI fragment of the β_2-microglobulin cDNA. EL4 is a cell line of *H-2^b* haplotype with high K^b surface expression. IG3, IB39, IB22, and IB34 are IC9 clones transfected with the isolated K^b gene. LG1, LG9, LB18, and LB19 are LTK$^-$ clones transfected with the same gene in an identical experiment.

described above for DNA-DNA hybridizations). Since β_2-microglobulin is known to be mandatory for surface expression of class I antigens, the amounts of β_2-microglobulin message in IC9, LTK$^-$, EL4, and some IC9 transfectants were monitored (Fig. 2B). No significant differences were observed, ruling out the possibility that β_2-microglobulin was the limiting factor. In untransfected IC9 cells, there was no K^b-specific mRNA detectable (Fig. 2A). Thus, the mechanism leading to the low surface expression of the transfected K^b gene does not seem to be involved in the down regulation of the silent endogenous class I genes.

The different regulation of endogenous and transfected class I genes was also evident after treatment with IFN-γ. Whereas IFN-γ did not lead to an induction of expression of the endogenous class I genes (Table 1), expression of the transfected K^b gene was enhanced (Table 2). Therefore, the cloned and transfected K^b gene underlies the same regulatory control by IFN-γ as the endogenous D^b gene (Tables 1 and 2) and other class I genes, further indicating that the cloned K^b gene contains an intact regulatory region.

Chromatin structure of the silent class I genes. The data presented so far show that the silent K^b gene is not mutated and can be expressed upon transfection. This raised the possibility that the silent class I genes are down regulated by a *cis*-acting mechanism at the level of the chromatin structure. In other studies it has been described that micrococcal nuclease (4) or DNase I (35) preferentially degrades the chromatin of active genes. We analyzed the chromatin structure of active and inactive class I alleles by limited digestions with both micrococcal nuclease and DNase I.

Cells (5×10^7) were lysed in 10 ml of lysis buffer (60 mM KCl, 15 mM NaCl, 15 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 14 mM β-mercaptoethanol, 5 mM sodium butyrate, 2 mM EDTA, 0.5 mM EGTA [ethylenediaminetetraacetic acid], 0.5 mM spermidine, 0.15 mM spermine, 0.1 mM phenylmethylsulfonyl fluoride, 0.8% Nonidet P-40 [pH 7.5]) for 10 min at 0°C. The nuclei were pelleted, suspended in lysis buffer, and centrifuged through 30% sucrose-1× lysis buffer. The nuclei were suspended in digestion buffer at a concentration of 10^8 nuclei per ml. Digestions with 10 U of micrococcal nuclease (S7 nuclease, Boehringer Mannheim) per ml were for 2, 5, 15, or 30 min at 37°C in a solution containing 0.25 M sucrose, 25 mM KCl, 25 mM NaCl, 10 mM Tris hydrochloride (pH 7.4), 5 mM sodium butyrate, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride. Digestions with 30, 60, 120, and 180 U of DNase I (grade I; Boehringer Mannheim) per ml were for 10 min at 0°C in a solution containing 60 mM KCl, 15 mM NaCl, 15 mM Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mM EGTA, 5% glycerol. The extent of digestion was determined by transferring the purified DNA to nitrocellulose membranes and hybridizing to specific probes. The DNA of active genes is degraded faster to fragments which are too small to bind to nitrocellulose membranes, resulting in a decreased hybridization signal.

In the case of IC9 cells, the chromatin of the silent K^b and K^k genes was indeed more slowly degraded by micrococcal nuclease than the chromatin of the active K^b and K^k genes in EL4 ($H\text{-}2^b$) or L929 ($H\text{-}2^k$) cells, respectively (Fig. 3A and C). As a control, we used the gene of the housekeeping protein dihydrofolate reductase, for which the kinetics of degradation were comparable in IC9, EL4, and L929 cells (Fig. 3B and D). For Fig. 3E, IC9 nuclei were digested with increasing amounts of DNase I and the purified DNA was redigested with the enzymes *Eco*RI, *Bgl*II, and *Xba*I. After

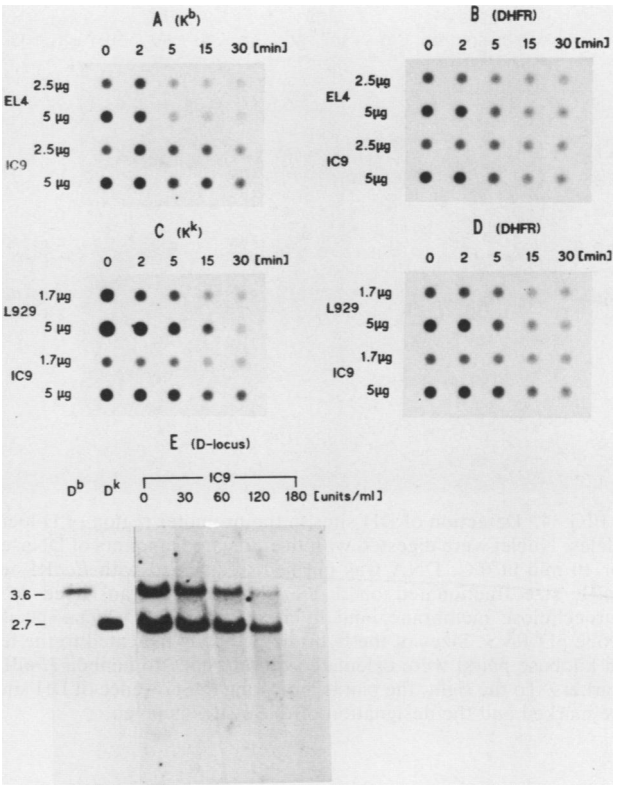

FIG. 3. Nuclease sensitivity of major histocompatibility complex class I genes in IC9, EL4, and L929 cells. Genomic DNAs in nuclei were digested with 10 U of micrococcal nuclease per ml for 2, 5, 15, or 30 min at 37°C (A through D) or with 0, 30, 60, 120, or 180 U of DNase I per ml for 10 min at 0°C (E). In panels A through D, the purified DNAs were dotted onto nitrocellulose; in panel e, the DNAs were recut with *Eco*RI, *Bgl*II, and *Xba*I, size fractionated on a 1% agarose gel, and then transferred to nitrocellulose. For hybridization, the following probes were used: a K^b-specific oligonucleotide (24) (A); a K^k-specific oligonucleotide (23) (C); a cDNA derived from the dihydrofolate reductase (DHFR) gene (17) (B and D); the D-locus-specific probe pD^{k}RVS (E) (for location, see Fig. 5). In panel B, identical amounts of the same DNA preparations as in panel A were used; in panel D, the filter used in panel C was stripped and rehybridized with the dihydrofolate reductase probe. In panel E, plasmids containing cloned D^b (1) and D^k (30) genes were used as markers.

hybridization to the D locus-specific probe pD^{k}RVS, two restriction fragments of different length appeared for the two D locus alleles as a result of the absence of the *Eco*RI site in the 5′ flanking region of the D^b gene (see Fig. 5). The hybridizing 5′ flanking fragment of the active D^b gene (3.6 kbp, located 0.5 to 4.1 kbp upstream from the transcription start site) was digested faster by DNase I than was the corresponding fragment of the D^k region (2.7 kbp, located 0.5 to 3.2 kbp upstream) (Fig. 3E). Thus, after digestion with micrococcal nuclease as well as with DNase I, the hybridization signal of the silent K^b, K^k, and D^k genes decreased significantly more slowly than the hybridization signal of the active genes (K^b in EL4, K^k in L929, D^b in IC9, and the dihydrofolate reductase gene).

DH sites are absent in the promoter region of the silent D^k gene. The chromatin structure of active genes is characterized by distinct sets of DNase I-hypersensitive sites (DH sites) generated by the displacement of nucleosomes. These sites are often found in the 5′ regulatory region of active

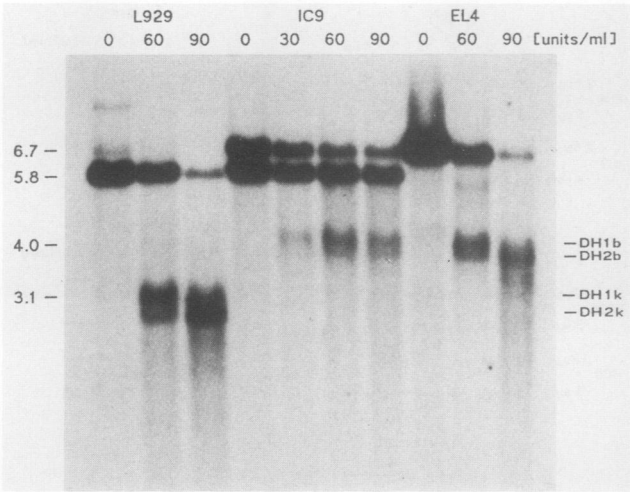

FIG. 4. Detection of DH sites in the promoter region of D-locus alleles. Nuclei were digested with the indicated amounts of DNase I for 10 min at 0°C. DNA was purified, redigested with *Eco*RI and *Bgl*II, size fractionated on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized to the D locus-specific probe pD^{k}RVS. Sizes of the hybridizing bands indicated to the left (in kilobase pairs) were calculated by reference to lambda-*Hin*dIII markers. To the right, the bands indicating the presence of DH sites are marked and the designation of these sites is given.

genes, where specific DNA-binding factors interact with the DNA, e.g., at the TATA box or the enhancer (13). To investigate potential hypersensitive sites in the promoter region of class I genes, we used the D locus-specific probe pD^{k}RVS. Digestion of genomic DNA with *Eco*RI and *Bgl*II and subsequent hybridization to this probe resulted in a band of 5.8 kbp, indicating the D^k gene (Fig. 4, L929 and IC9), or in a band of 6.7 kbp, indicating the D^b gene (Fig. 4, EL4 and IC9). The greater size of the D^b band was due to the absence of the *Eco*RI site in the 5′ flanking region of the D^b gene (Fig. 5). Therefore, it was possible to differentiate between the

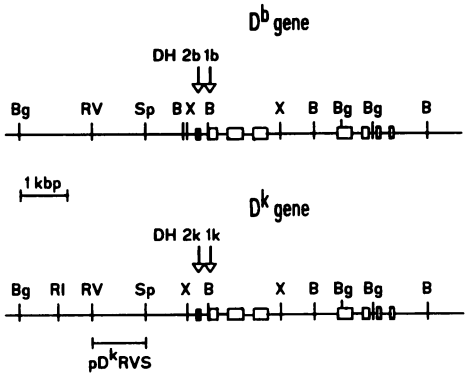

FIG. 5. Location of DH sites in the promoter region of D-locus alleles. Positions of DH sites (shown by the arrows above the map together with their designations) were determined by calculating the size of the bands shown in Fig. 4. Indicated restriction sites were in part derived from the works of Stephan et al. (30) and Watts et al. (34). B, *Bam*HI; Bg, *Bgl*II; RI, *Eco*RI; RV, *Eco*RV; Sp, *Sph*I; X, *Xba*I. Symbols: □, exons; ■, location of the enhancer-interferon response sequence. The location of the D locus-specific probe pD^{k}RVS is indicated by a bar below the map.

two D-locus alleles. When nuclei of EL4 and L929 cells were incubated with DNase I (see above) prior to digestion with *Eco*RI and *Bgl*II, two additional bands appeared, indicating DH sites (designated DH1b and DH2b for the D^b gene and DH1k and DH2k for the D^k gene) (Fig. 4). Calculation of the size of these two bands resulted in the approximate location of the DH sites at the transcription start site (DH1b and DH1k) and at the enhancer-interferon response sequence (DH2b and DH2k) of the D locus genes (Fig. 5). When nuclei of IC9 cells were digested with DNase I, only two DH sites corresponding in size of those of the D^b gene of EL4 cells (DH1b and DH2b) could be detected. These bands were obviously derived from the active D^b allele in the IC9 cells. Bands indicating the hypersensitive sites of the D^k gene in L929 chromatin were absent from IC9 (Fig. 4).

In summary, we have shown that the class I deficiency in IC9 cells is not caused by the action of a *trans*-acting factor, as has been suggested for other class I-deficient tumor cell lines (3, 11, 14, 19). We base this conclusion on the following findings: (i) in somatic hybrids with an H-2-positive cell line, the expression of the silent IC9 class I genes was not induced and expression of the active class I genes of the fusion partner was not repressed; (ii) the three known relevant class I-specific DNA-binding factors which bind to the main regulatory region of class I genes could be detected in nuclear extracts of IC9 cells; (iii) the silent K^b gene which was cloned from IC9 cells could be expressed after transfection in IC9 cells. This finding also excluded the possibility that major mutations within the K^b gene prevented its expression. Thus, we concluded that class I deficiency was caused by a regulatory mechanism acting in *cis*. Respective possibilities included an alteration in the methylation pattern or in the chromatin structure. In the case of IC9 cells, treatment with the demethylating reagent 5-azacytidine was ineffective and no differences in the methylation pattern between the silent class I genes of IC9 and the class I genes of H-2-positive cell lines could be observed (results not shown).

We therefore favored the possibility that a more condensed chromatin structure prevented expression of the K^b, K^k, and D^k class I genes in IC9 cells. Indeed, we could find a decreased nuclease sensitivity of the inactive class I genes in nuclei of IC9 cells, in comparison with the active class I genes of EL4 and L929 cells. Another striking difference between the chromatin structure of active and inactive D-locus alleles was the absence of DH sites in the 5′ flanking region of the silent D^k gene in IC9 nuclei. This indicates that the DNA of the D^k regulatory region is associated with nucleosomes rather than with the class I-specific DNA-binding factors. This is of special importance because these factors are present in IC9 nuclei and can interact with the class I regulatory region, as indicated by the presence of DH sites in the 5′ flanking region of the D^b gene. Thus, the down-regulatory mechanism of class I genes in IC9 cells primarily interferes at the level of the chromatin structure. We suggest that the silent class I genes are packaged in a more condensed chromatin structure, thus preventing the class I-specific DNA-binding factors from interacting with their target sequences. This inactivation mechanism not only discriminates between different class I loci (D^b versus K^b) but also between two alleles of the same gene (D^b versus D^k).

In conclusion, we have shown that changes in the chromatin structure participate in the inactivation of the class I genes in the IC9 fibrosarcoma. At this stage it cannot be decided whether changes in the chromatin structure precede

the pathway of a cell to malignancy or accompany it. However, in both cases this mechanism appears to provide a selective advantage to the class I-negative tumor cells because it results in a decrease of their immunogenicity.

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